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CHARACTERIZATION OF FUNGAL EXTRACTS FROM *TRICHODERMA* ISOLATES: THEIR EFFECTS AGAINST COFFEE WILT PATHOGEN (GIBBERELLA *XYLARIOIDES)*

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ABSTRACT: The current research work was designed to evaluate, test, and characterize effective antifungal extracts from *Trichoderma* isolates against coffee wilt pathogen (*Gibberella xylarioides)*. For extraction of antifungal extracts from fungal mycelium different organic solvents, *viz.*, chloroform, ethanol, methanol, ethyl acetate, n-hexane and butane were used. A direct bioautographic procedure was conducted, involving spraying suspension of *Fusarium xylarioides* on Thin Layer Chromatography (TLC) plates developed in solvents of varying polarities to detect a number of antifungal substances present in the extracts. Moreover, *in vitro* antagonistic bioassays were performed to evaluate and determine the potentiality of *Trichoderma* isolates as biocontrol agents against *F. xylarioides*. Antifungal extracts were successfully extracted from malt extract agar medium with all organic solvents used except from hexane. Bioautography assay revealed 60 zones of inhibition spots and the highest inhibition zone was observed in AUT5 (51 mm) and AUT6 (44 mm) with ethanol extract at R_f value of 0.43. In *in vitro* bioassay, the highest mean inhibitory effect on the growth of the pathogen was achieved by AUT2 (77.4%) isolate in dual culture. In general, TLC-directed bioautography assay was found to be useful in isolating active compounds with antifungal activity and all *Trichoderma* isolates significantly reduced mycelial growth of the test pathogen compared to the control under *in vitro* condition.

Key words/phrases: Antifungal compound(s), bioassay methods, bioautography, organic solvents, thin layer chromatography

INTRODUCTION

Coffee is a tropical and subtropical crop grown in more than 80 countries with a total production close to 108 million tons per year (Wrigley, 1988). The genus *Coffea* is endemic to Africa and a number of species are described in West, Central and East Africa. Due to disease constraints, poor management practices and other factors such as yield, quality and growth habits, only two species are nowadays commercially grown worldwide; *viz.*, *C. canephora* (Robusta) in lowlands and *C. arabica* (Arabica) in highlands (Hindorf and Omondi, 2011; Musoli *et al*., 2001). However, coffee remains the most important export crop for many African countries both in terms of earnings and its impact on socio-economic life of the rural folk engaged in its production (Flood, 2009).

Ethiopia is the primary gene center for Arabica coffee (*Coffea arabica* L.) (Tefesetewold Biratu, 1995). Coffee contributes 40–60% of the foreign exchange earnings to the country and nearly 25–30% of the Ethiopian population depends directly or indirectly on coffee for its livelihood, getting involved in the production, processing and marketing as the major contribution to the development of the national economy (Girma Adugna *et al*., 2009).

Coffee wilt disease (CWD) has been limiting coffee production in most parts of Eastern and Central Africa and continues to spread to the neighboring region at an alarming rate. The disease is greatly distributed in many parts of Eastern, and Central African countries such as Ethiopia, Uganda, Democratic Republic of Congo, and Ivory Coast (Rutherford, 2006). The incidence of this disease in Ethiopia is reported to be 60%, with significant yield losses due to

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severe damage and ultimate death of millions of coffee trees (Girma Adugna *et al*., 2001; Girma Adugna, 2004). The disease is responsible for reduction in the production of coffee beans, since it severely attacks the vascular system of the plant causing wilting and eventually dieback (Tesfaye Alemu, 2012). It has been reported that the high cost of pesticides, emergence of fungicide-resistant pathogen biotopes and other social and health related impacts on the environment have increased interest in agricultural sustainability and integrated disease management (Cook *et al*., 1996; Vander Vossen, 2005). Thus, there is a need for sustainable solutions such as biological control agents and integrated disease management to reduce coffee wilt disease problems that could provide effective control, while minimizing cost and rate of application antagonists for establishment of sustainable agricultural development and ecofriendly/dynamic farming system for human health and the environment.

Species of the genus *Trichoderma* have been proven as effective biocontrol agents (BCAs) of soil-borne plant pathogens (Jensen and Wolffhechel, 1993). The genus was described by Persoon almost 200 years ago and consists of anamorphic fungi isolated primarily from soil and decomposing organic matter (Harman *et al*., 2004). To date, most of the studies on *Trichoderma* species have been conducted with respect to their antifungal activity as biological control agents. Their efficacy has been demonstrated for the management of fungal diseases and nematode under greenhouse and field conditions (Siddiquee *et al*., 2009). The modes of action as biological control agents include mycoparasitism, antibiosis, competition, lytic enzyme activity production and induction of plant defense (Howell, 2003). Understanding the mechanisms involved in the antagonistic effect of *Trichoderma* species against plant pathogen are becoming very important in the selection of suitable biocontrol agents for effective and safe utilization (Negash Hailu and Tesfaye Alemu, 2009).

Thus, the objective of the current research work was to evaluate and characterize effective antifungal extracts from *Trichoderma* isolates against coffee wilt pathogen (*Gibberella xylarioides*). In addition, in this study *in vitro* activity, bioassay methods and thin layer chromatography (TLC) bioautographic techniques in evaluation and determination of antifungal extracts from *Trichoderma* isolates against the test pathogen were also employed.

MATERALS AND METHODS

Trichoderma isolates and test pathogen

Pure cultures of *Trichoderma* isolates and CWD (*F. xylarioides*) were obtained from Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University (AAU), and Jimma Agricultural Research Institute, respectively. All the isolates were designated as AUT1-AUT6, in which AUT stands for Addis Ababa University *Trichoderma* isolate. Characterization and *in vitro* antagonistic evaluation of potential antifungal compounds were conducted in the above two Mycology and Applied Microbiology Research Laboratories while solvent extraction and purification by chromatographic separation and detection were performed in the Analytical Laboratory, Department of Chemistry, AAU, from 2011 to 2012.

Antagonism of Trichoderma isolates against Fusarium xylarioides

Six different isolates of *Trichoderma* were individually tested for their antagonistic activities against *F. xylarioides* at Jimma Agricultural Research Institute using the dual culture technique (Negash Hailu and Tesfaye Alemu, 2009). Fungal agar blocks of 5 mm diameter of *F. xylarioides* and *Trichoderma* isolates were taken from pure cultures of 8 days old and placed on plates of potato dextrose agar (PDA) at a distance of 5 cm between the *F. xylarioides* and the *Trichoderma* isolates (Negash Hailu and Tesfaye Alemu, 2009). Five plates were prepared as replicates for each isolate. Plates inoculated with *F. xylarioides* alone served as control. Plates were incubated at 25°C for 7 days. Radial growth of *F. xylarioides* was measured every 2 days after inoculation and the plates were kept in incubator. Clear zone of inhibition was also determined by measuring the mycelial growth between the colony margins of the *F. xylarioides* and *Trichoderma* isolates. Percentage inhibition of radial growth (PIRG) was calculated following the formula suggested by Rita and Tricita (2004):

$$\text{PIRG} = \frac{\text{R1} - \text{R2}}{\text{R1}} \times 100$$

where, R1 (mm) is the colony diameter of the test pathogen in the control and R2 (mm) is the colony diameter of the pathogen with antagonistic interaction.

Antagonism was assessed in semi-quantitative means (Rita and Tricita, 2004): > 85 PIRG indicating very high antagonistic activity, 61–85 PIRG indicating high antagonistic activity, 51–61 PIRG indicating moderate antagonistic activity, < 50 PIRG indicating low antagonistic activity and 0 PIRG indicating no activity.

Antifungal activity of crude extracts of Trichoderma isolates

Crude extracts obtained from both solid and liquid culture media were mixed and tested against the fungal pathogen after 250, 500, 750 and 1000 µl of crude sample was amended with 25 ml of PDA media after autoclaved before pouring of the medium. The mycelial of the test pathogen was seeded on the solid medium. Culture medium, without crude extracts, was also seeded with the test pathogen and incubated for 7 days at 25°C to serve as control. The antifungal activity of the crude extract against the test pathogens was recorded as the radial growth inhibition (Kaushal *et al*., 2013).

Extraction method for antifungal compounds from Trichoderma isolates

Extraction of antifungal compounds from solid culture medium

To test the production of inhibitory substances/-extracts on solid medium, *Trichoderma* isolates and *Fusarium xylarioides* were grown on malt extract agar (MEA) incubated at 25°C for 7 days.

For extraction of antifungal compounds from MEA medium different organic solvents; *viz*., chloroform ($CHCl_3$), ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc), hexane (n-C_6H_{14}) and butanol (BuOH) were used. Extraction of antifungal extracts was employed by using rotary evaporator, taking into consideration the boiling point of various solvents (($CHCl_3$, 56°C), (EtOH, 89°C), (MeOH, 65°C), (EtOAc, 88°C), (n-C_6H_{14}, 69°C) and (BuOH,118°C)). To harvest and extract antifungal compounds from solid culture medium, three Petri dishes were used as replicates for each of the *Trichoderma* isolates. During harvesting process, 25 ml/Petri dish of each organic solvent was added to the 7 days old cultures of *Trichoderma* isolates in flasks and placed on a shaker at 121 rpm for 30 min and finally filtered using Whatman No.1 filter paper under aseptic condition. The culture filtrate was then extracted by using rotary evaporator and further purification of the crude extract was achieved by use of Thin Layer Chromatography (TLC). Moreover, the crude extracts were tested against the test pathogen by using bioassay methods (Hamburger and Cordell, 1987).

Extraction of antifungal compounds from liquid culture medium

The *Trichoderma* isolates were cultured in potato dextrose broth (PDB) at 25°C for 21 days. After 21 days of incubation, each broth culture was filtrated twice by using Whatman No.1 filter paper under aseptic condition and centrifuged at 5,000 rpm for 20 min to make it cell free (Aneja, 2005). To 100 ml of culture filtrate, 25 ml of each solvent was added to each broth culture filtrate, shaken for 5 min and the solvent and aqueous layer were separated using solvent fractioning (Howell, 2003). The crude extract of *Trichoderma* isolates was then tested against the test pathogen by using bioassay methods (Hamburger and Cordell, 1987).

Methods of assaying for antifungal compounds

Detection and assaying of active antifungal compound(s) by TLC

Thin Layer Chromatography (TLC) was used for separation of extracts and fractions, and to screen the qualitative purity of the compounds using TLC aluminum sheets silica gel 60 F_{254} pre-coated 20×20 cm as described by El-Mougith *et al*. (1986). Attempts were made to isolate the active extracts of the antifungal compounds using TLC. Standard chromatograms of fungal extracts were prepared by applying 20 µl solution to a silica gel of the TLC plate. For successful detection and separation of antifungal metabolites, different solvent systems were optimized, tested and determined using TLC (Aluminum sheet Silica gel 60 F_{254}, Merck) (Table 1).

Table 1. Solvent mixtures screened for TLC analysis of antifungal extracts from *Trichoderma* isolates.

Solvent System (SS)	Composition of Solvent System	Ratio (v/v)
1	Hexane : Benzene : Chloroform	4: 2 : 4
2	Acetone: Acetic Acid: Toluene	1: 1: 8
3	Hexane : Benzene: Chloroform: Toluene	5: 4: 5: 6
4	Hexane: Benzene: Methanol	3: 2: 5
5	Hexane: Ethanol: Methanol	5: 3: 2
6	Hexane: Benzene: Chloroform	2: 5: 3
7	Hexane: Toluene: Chloroform	3: 4: 3

Thin Layer Chromatography was performed in sealed glass chambers. Solvents were added separately into each chamber to a depth of about 2 cm. A piece of filter paper (No. 1) was placed at the center of the chamber and was left for 30 min to saturate the chamber before inserting a TLC plate. TLC plates were cut to an appropriate size (10 cm × 10 cm) and 20 µl crude extracts were spotted on a line, 2 cm from the bottom edge. The TLC plate was then immersed in the closed chamber and left until the solvent almost reached the opposite side of the plate. Separated spots, travelling different distances on the plate, were then detected by their ultra violet (UV) absorbance at the wavelength 254 nanometer (nm) and/or by spraying the TLC plates with spraying reagents, tetrazolium salt, followed by heating at 110°C (Geetha *et al*., 2003). The distances of the spots and the solvent front were measured from the final to initial readings to calculate the relative mobility or retardation factor (R_f), using the equation given below. Active spots were located and detected from developed TLC plates by direct bioautography.

$$R_f = \frac{\text{Distance moved by the crude sample from the origin to spot centre}}{\text{Distance moved by solvent systems from the origin to solvent front}}$$

The use of TLC *with direct bioautography for antifungal analysis*

The direct bioautography was applied by transferring the test pathogen directly on TLC plate. The extracts were taken from *Trichoderma* isolates grown on MEA medium (solid medium) after seven days of incubation at 25°C. A diffusion process transferred active antifungal extracts from the stationary phase to the agar layer, which contains the *F. xylarioides*. After incubation, the plate was sprayed with a tetrazolium salt, such as 2, 3, 5-Triphenyltetrazolium bromide (TTB), which is converted to a formazan dye by the micro-organism (Suleiman *et al*., 2010).

Screened solvent mixtures with optimum R_f were added into a chamber to a depth of 2 cm or 100 µl and each of the extracts was applied on the spots of two TLC plates with a micropipette and subsequently developed using appropriate solvent system. Organic solvents, which cause inactivation of enzymes or death of living organisms, were completely removed before biological detection. About 25–50 ml inoculum solution spray was prepared containing 1×10^6 spores/ml of 7-day test fungus, *F. xylarioides*. The plates were sprayed lightly three times with concentrated spore suspension and incubated on a sheet of filter paper in a moist Petri dish for 24 hours in darkness at 25 ± 2°C and then sprayed with 2 mg/ml solution of 2, 3, 5-triphenyltetrazolium bromide (TTB) as a growth detector (Silva *et al*., 2006). A moist chamber was further incubated for 5 days to allow the fungal growth of the pathogen. Finally, the TLC plate was dried and visualized using UV-light at 254 nm. The diameter of the inhibition zone was measured in millimetre (mm) and the active spots were observed under UV light. The antifungal activity of the separated compounds was recorded as absence of growth over the active extract (white clear zone). The R_f of the inhibition zones on the plate (plate B) was compared with the R_f of the reference chromatogram (plate A) to determine the R_f of the active compound.

Effect of non-volatile compounds from Trichoderma isolates on the radial growth of F. xylarioides

The non-volatile (culture filtrate) of *Trichoderma* isolates was investigated to determine their influence on the mycelial growth of the test fungus (*F. xylarioides*) on PDA. The filtrates that were maintained under aseptic conditions also amended with PDA to make 5, 10, 20 and 40% (v/v) concentration in Petri plates (Negash Hailu and Tesfaye Alemu, 2009). The solidified agar plates, in triplicates, were inoculated at the centre with 5 mm diameter mycelial disc of pathogen and incubated at 25 ± 2°C for 7 days. The colony diameter was measured after 7 days of incubation and the percent inhibition of mycelial growth was calculated according to the equation suggested by Rita and Tricita (2004).

RESULTS

In vitro evaluation of antagonistic activity of Trichoderma isolates on Gibberella xylarioides

The experimental results showed that that the six isolates of *Trichoderma* tested were able to inhibit the growth of *G. xylarioides* under *in vitro* experiment (between 59.5% and 77%) after 7 days of incubation (Fig. 1). The highest mean inhibitory effect on the growth of the test pathogen was achieved by AUT2 (77%) followed by AUT3 (72.9%) and AUT1 (67.6%), while AUT6 isolate showed the lowest (59.6%) mean inhibitory effect against *F. xylarioides* after 7 days

of incubation. AUT1, AUT2 and AUT3 isolates of *Trichoderma* were rapidly grown in the form of powdery and spread widely throughout the Petri plates (Fig. 1). These isolates failed to develop inhibition zones, since they grew in the form of wide spread powders and occupied all the spaces. The interaction was due to the competition for space and nutrients rather than forming inhibition zones (Fig. 1). On the other hand, AUT4 and AUT5 isolates produced zones of inhibition, by inhibiting *F. xylarioides* (Fig. 1). There was no complete overgrowth on the test pathogen while AUT6 isolate secreted some oily substances towards the test pathogen. It formed intermediate zones of inhibition as well as overgrew on the hyphae of *F. xylarioides*. The results of this study indicated that there were differences in per cent of inhibition of the mycelial growth rate of *F. xylarioides*, which resulted from metabolites released by *Trichoderma* isolates (Fig. 1).

Extraction of antifungal extracts from Trichoderma isolates

Among the organic solvents used for separation processes, only chloroform and hexane were found to be the best solvents that formed layers readily but similar layers were not observed for the other solvent mixtures. However, all the organic solvents used have enabled extraction of the crude compounds from the antagonist except n-hexane.

Antifungal activity of crude extracts

The inhibition diameters for the crude extracts were computed against the test pathogen as given in Table 2. Accordingly, isolate AUT3 with hexane extract and AUT4 with butane extract obtained from solid culture medium showed complete inhibition (100%), while isolate AUT5 with ethanol extract and AUT6 with methanol extract, exhibited a moderate to high antifungal activity over the tested pathogen. The crude chloroform extract showed least potent inhibitory effect on the growth of *F. xylarioides* (2.7%) at a concentration of 750 µl (Table 2).

Analysis of crude extracts and detection of R_f values of antifungal extracts on TLC

In this study, 14 solvent systems were screened of which seven were selected for further TLC and bioautographic analyses (Table 3). Only three extraction solvents exhibited the optimum R_f values: $CHCl_3$, EtOH and BuOH. Methanol and ethyl acetate crude extracts were not eluted sufficiently and their R_f values approach zero and/or 1 (Table 3). This may be due to the high affinity of crude extracts to the stationary phase than the mobile phase in which the crude sample remain at the origin (R_f value was too low). On the other hand, if the extract had high affinity for the mobile phase than stationary phase, the crude samples would have reached the solvent front, the condition related to the highest R_f value.

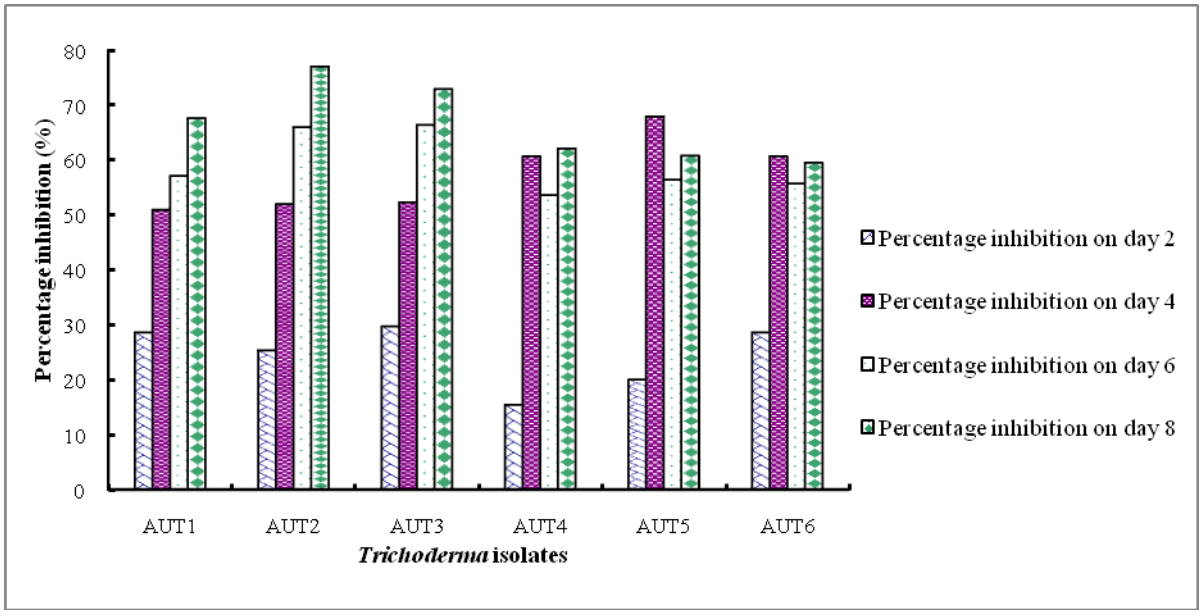


Figure. 1. Percentage inhibition of radial growth of *Trichoderma* isolates against *F. xylarioides*. (**AUT** = Addis Ababa University *Trichoderma* isolates).

Table 2. Antagonistic activity of crude extracts from *Trichoderma* isolates at different concentrations.

Trichoderma Isolates	Extracts	Concentration of crude extract (µl)							
		250	PIRG (%)	500	PIRG (%)	750	PIRG (%)	1000	PIRG (%)
AUT1	Chloroform	15	18.9	14	24.3	18	2.7	16	13.5
AUT2	Ethyl acetate	8	56.8	11	40.5	10	46.0	5	73.0
AUT3	Hexane	CI	0	CI	0	CI	0	CI	0
AUT4	Butane	CI	0	CI	0	CI	0	CI	0
AUT5	Methanol	10	46.0	6	67.6	5	73.0	5	73.0
AUT6	Ethanol	6	67.6	7	62.2	5	73.0	5	73.0
Control					18.5				

Note: PIRG = Percentage inhibition of radial growth; CI = Complete inhibition.

Table 3. Solvent systems and R_f values of the antifungal extracts on TLC analysis.

Solvent System (SS)	Composition of solvent system	Ratio of SS (V/V)	Type of Extract	R_f values of antifungal compounds					
				AUT1	AUT2	AUT3	AUT4	AUT5	AUT6
SS_1	n-Hexane:	3: 4: 3	$CHCl_3$	0.89	0.80	0.88	0.84	0.85	0.75
	Toluene:		EtOH	0.80	0.80	0.80	0.80	0.80	0.84
	Chloroform		BuOH	0.80	0.62	0.57	0.77	0.65	0.58
SS_2	n-Hexane:	4:2:4	$CHCl_3$	0.40	0.88	0.46	0.87	0.44	0.48
	Benzene:		EtOH	0.16	0.14	0.14	0.16	0.16	0.16
	Chloroform		BuOH	0.50	0.50	0.54	0.54	0.57	0.57
SS_3	Acetone:	1:1:8	$CHCl_3$	0.82	0.84	0.86	-	0.84	0.84
	Acetic Acid:		EtOH	0.66	0.62	-	-	-	0.50
	Toluene		BuOH	0.76	0.78	0.76	0.78	0.78	0.78
SS_4	n-Hexane:	5:4:5:6	$CHCl_3$	0.67	0.64	0.63	0.57	0.64	0.69
	Benzene:		BuOH	0.63	0.65	0.73	0.75	0.79	0.76
	Chloroform:								
	Toluene								
SS_5	n-Hexane:	3:2:5	BuOH	0.72	0.76	0.72	0.68	0.62	0.58
	Benzene:								
	Methanol								
SS_6	n-Hexane:	5:3:2	EtOH	0.58	0.58	0.60	0.87	0.88	0.80
	Ethanol:								
	Methanol								
SS_7	n-Hexane:	2:5:3	$CHCl_3$	0.35	0.36	0.36	0.40	0.35	0.36
	Benzene:		EtOH	0.18	0.22	0.25	0.83	0.83	0.20
	Chloroform								

Note: _ indicates R_f = 0 or 1; $CHCl_3$= chloroform; EtOH=ethanol; MeOH=methanol; BuOH= butanol

The use of TLC with direct bioautography for antifungal analysis

Fifteen TLC plates for seven solvent mixtures were tested in the bioautographic procedure (Table 1). Many compounds present in the extracts inhibited the growth of *F. xylarioides* (Table 4). AUT5 appeared to be the *Trichoderma* isolate having crude extract with the highest number of inhibition spots compared with the other isolates tested against the test fungus whereas the lowest number of inhibition spots were observed in AUT6 (Table 5). On the other hand, the highest inhibition zone was observed in AUT6 (51 mm) (Table 5) with ethanol extract (SS1) at R_f (0.43) followed by AUT5 (44 mm) with ethanol extract (SS1) at R_f value of 0.43 and AUT1 (41 mm) with butane extract (SS5) at R_f value of 0.52. The lowest inhibition zone on bioautography was 4 mm (Table 5).

Table 4. Inhibition of growth (diameter) on bioautographic TLC plates by 3 extracts of AUT4 against *F. xylarioides* (A= R_f values of reference chromatogram, B = R_f values of active compound on bioautogram).

Solvent Systems	Extracts	R_f Values		Inhibition Diameter (mm)	Active spots	Total Active spots
		A	B			
SS_1	EtOH	0.8	0.43	44	1	
	BuOH	0.65	0.89	12	1	
SS_2	$CHCl_3$	0.44	0.19	14	1	
	EtOH	0.16	0.77	10	1	
SS_3	$CHCl_3$	0.84	0.93	10	1	
	BuOH	0.78	0.62	17	1	12
SS_4	$CHCl_3$	0.64	0.66	4	1	
	BuOH	0.79	0.77	4	1	
SS_6	EtOH	0.88	0.88	5	1	
SS_7	$CHCl_3$	0.35	0.4	20	1	
	EtOH	0.83	0.24, 0.83	14, 5	2	

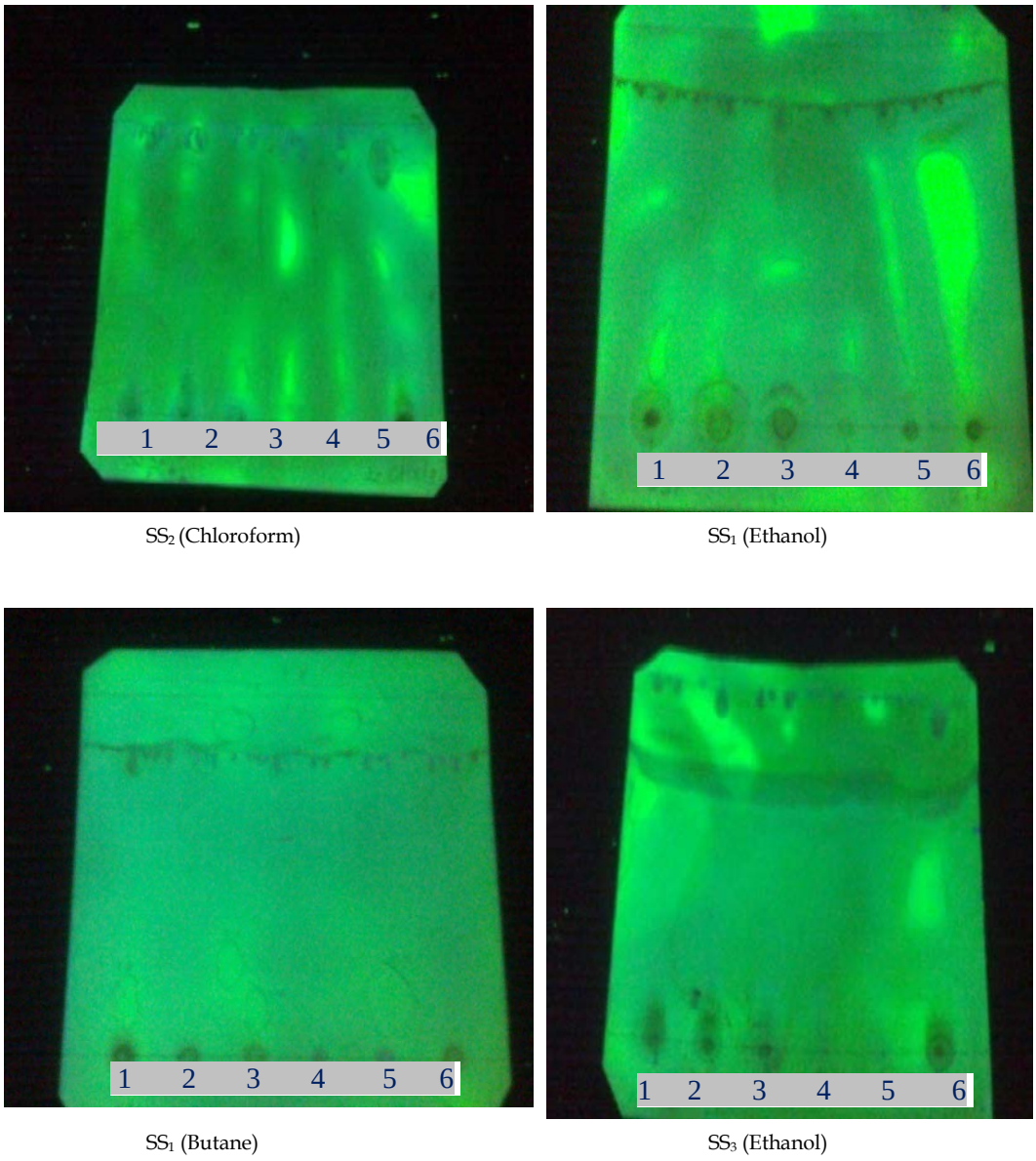
Note: Extracts as in Table 3.

Table 5. Inhibition of growth (diameter) on bioautographic TLC plates by 3 extracts of AUT5 against *F. xylarioides*.

Solvent Systems	Extracts	R_f Values		Inhibition Diameter (mm)	Active spots	Total Active spots
		A	B			
SS_1	EtOH	0.84	0.43	51	1	
SS_2	$CHCl_3$	0.48	0.54, 0.77	10, 13	2	
	EtOH	0.16	0.54	12	1	
SS_3	$CHCl_3$	0.84	0.47	17	1	8
SS_5	BuOH	0.58	0.37	32	1	
SS_6	EtOH	0.80	0.86	5	1	
SS_7	$CHCl_3$	0.36	0.40	13	1	

Note: Extracts as in Table 3; A and B as in Table 4.

It has been demonstrated in this study, that there were 60 zones of inhibition detected with different extracts, test fungus and solvent systems used in *Trichoderma* extracts. Different solvent systems were optimized and analyzed to determine which TLC solvent system separated the highest number of active compounds. Solvent system 1 (SS1) was the best solvent (15 active spots) followed by SS2 (14 active spots) to separate the active antifungal compounds from *Trichoderma* isolates (Fig. 2). This implies that polar systems separated less active compounds compared to the non-polar systems. Moreover, the best extracting solvent of active antifungal compound was chloroform (25 zones of inhibition) followed by ethanol (20 zones of inhibition) and butane (15 zones of inhibition). With R_f values of the reference chromatogram (A), active antifungal compound on bioautogram (B) inhibition zones (mm) and number of active spots present in different extracts eluted with different solvents from AUT5 and AUT6 against *F. xylarioides* are presented in Tables 4 and 5, respectively.



SS_2 (Chloroform) SS_1 (Ethanol)

SS_1 (Butane) SS_3 (Ethanol)

Figure 2. Direct TLC Bioautography for the detection of antifungal activity against test pathogenic fungus *F. xylarioides*. (SS= solvent system, 1 = AUT1, 2 = AUT2, 3 = AUT3, 4 = AUT4, 5 = AUT5, 6 = AUT6).

In general, chloroform was the best extraction solvent with more antifungal extracts from *Trichoderma* isolates. Most compounds were extracted in low polarity solvent (chloroform), few in high polarity solvent (ethanol) and least in intermediate polarity solvent (butane). A number of isolates used in this study showed promising biological activity.

Effect of non-volatile antifungal extracts on radial growth of F. xylarioides

The culture filtrate (non-volatile compound) from all the *Trichoderma* isolates exhibited growth inhibition (Fig. 3). It was observed that culture filtrate from AUT3 showed high antagonistic activity (78.4%) at a concentration of 40%, whereas AUT2 exhibited 72.9% (Fig. 3). The minimum inhibitory concentration observed in isolate AUT5 and AUT6 was 56.8% for both isolates at 5% concentration of culture filtrate. In general, maximum and minimum inhibitions were recorded in isolate AUT3 containing 40% culture filtrate and in isolate AUT1 containing 20% of the culture filtrates, respectively (Fig. 3).

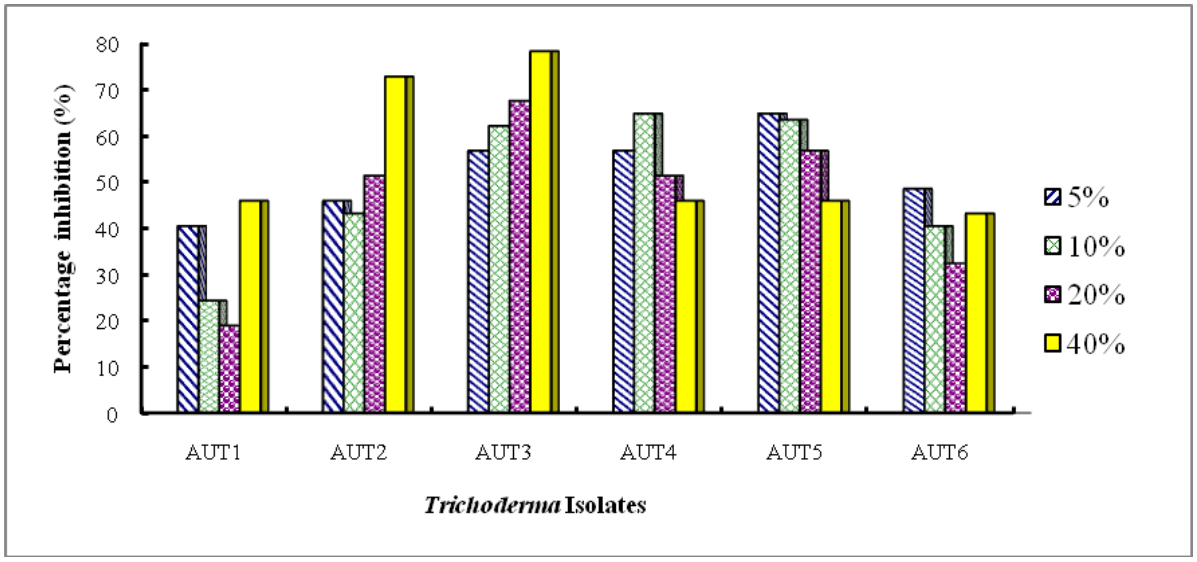


Figure 3. Percentage inhibition radial growth of *F. xylarioides* with culture filtrate (non-volatile compounds) obtained from *Trichoderma* isolates.

DISCUSSION

Trichoderma species biocontrol potential was the result of a number of qualities, which include antagonism, antibiosis and lytic enzymes production that digest the cell wall (Kamal *et al*., 2009; Hanson and Howell, 2004). Almost all *Trichoderma* isolates tested showed high biocontrol activity against the test pathogen, which is in agreement with a previous report on fungal biocontrol agents (Rita and Tricita, 2004). Variations in the inhibitory potential may be due to the differences in the quantity and quality of the inhibitory substances produced by the antagonists as reported by Hanson and Howell (2004) who demonstrated that concentration of the secondary metabolites produced by *Trichoderma* species, determines inhibitory activity of these compounds.

Rapid growth of *Trichoderma* was observed to be an important condition in competition with plant pathogenic fungi for space and nutrients. Competitiveness was based on rapid growth and the production of various asexual produced conidia and chlamydospores (Chet, 1987). *Trichoderma* isolate AUT1 and AUT2 were the quickest of all isolates in crossing the zone of inhibition and parasitizing *F. xylarioides*. On agricultural fields, dominance of biocontrol agent through its high growth rate and offensive mechanisms against pathogen was decisive in manifestation of disease control (Zimand *et al*., 1996). Parasitism by *Trichoderma* isolates was its powerful weapon in the destruction of the pathogens. Accordingly, *Trichoderma* isolates must overwhelm pathogen before the pathogen proliferation and infection of the germinating plant seeds.

In dual culture, AUT2 and AUT3 *Trichoderma* isolates inhibited the radial growth of *F. xylarioides* with the largest growth reduction of 77% and 72.9%, respectively. These *Trichoderma* isolates formed coiled structures around the hyphae of the test pathogen (*F. xylarioides)* which was observed under microscope. This coiling is a characteristic of the interaction between mycoparasitic and phytopathogenic fungi leading to penetration of the cell wall (Harman *et al*., 2004). In general, *Trichoderma* isolates, which inhibited the growth of the test fungus, could be used in the biological control of the fungal diseases caused by *F. xylarioides*.

Considering differences in growth of *F. xylarioides* under direct intense influence of the *Trichoderma* isolates, due to rapid spread of *Trichoderma* isolates on dual culture plate with the advancement of interaction, the test pathogen was almost encircled by *Trichoderma* isolates. Even the distal side of *F. xylarioides* colony came under influence of *Trichoderma* isolates and no side of *F. xylarioides* colony was free from influence of *Trichoderma* isolates. It has been indicated that the microscopic observation of *Trichoderma* isolates and *F. xylarioides* interaction on slide culture showed that cell wall appeared thick and dark in *F. xylarioides* mycelia. *Trichoderma* isolates showed heavy sporulation

where it encountered metabolites of *F. xylarioides*. *Fusarium xylarioides* mycelia away from *Trichoderma* isolates showed smooth, hyaline and normal morphology.

As *Trichoderma* isolates are a promising antagonistic for major soil borne diseases, studies on their antifungal substances have extensively made in order to find out the best active compound and the mode of action (Kimani *et al*., 2002). In this study, different organic solvents separated antifungal extracts and the highest antifungal activity was achieved with chloroform, ethanol and butane on TLC coupled with bioautography whereas hexane and butane crude extracts were highly significant in reducing the mycelial growth of the test pathogen under *in vitro* condition. The type and composition of the eluent in TLC is one of the variables influencing the separation processes, since it shows differences in chemical composition of the antifungal extracts. The solubility, affinity and the resolution of the solvent system greatly determine the R_f values of the crude extracts (Tarman, 2011).

An important factor in quantifying the movement of a compound on a stationary phase with a certain solvent system is the retardation factor (R_f value). Crude extracts of *Trichoderma* isolates were subjected to TLC bioautography to measure their retention frequency, inhibition zones and to count the active band(s). According to the findings of this study, the highest inhibition zone on bioautographic assay was observed in AUT5 and AUT6 with ethanol extract at R_f value of 0.43 (Table 4 and 5). Since, the R_f value is constant for the same compound under defined conditions, the presence of clear spots with the same R_f value may mean that the same compounds are probably responsible for the antifungal activity. TLC bioautographic methods combine chromatographic separation and *in situ* activity facilitating the localization and target directed isolation of active constituents in a mixture (Shahverdi *et al*., 2007).

In some cases, *F. xylarioides* did not grow well and it was difficult to detect inhibition zones. The inhibition was only detected early during incubation. Subsequent growth of mycelia on top of an active band made it difficult to see the inhibition. There were a few cases where the test fungus did not grow well on certain parts of the bioautogram, making it difficult to evaluate the number of antifungal compounds (Woo *et al.,* 2005). The non-activity of the other *Trichoderma* isolate extracts by using bioautography assays could be explained by a weak activity of the extracts against the test pathogenic fungus with the disruption of synergism between active constituents caused by TLC separation, or the low concentration of the active compounds in the crude extract under the tested conditions. In other cases, there were growths but no inhibitions were observed. The non-activity of these extracts in bioautography may possibly explained by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC separation (Hamburger and Cordell, 1987).

The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the fungus because of the presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce TTB to a purple-red colour (Suleiman *et al*., 2010). In the presence of active compounds on the chromatograms, the growth of the organism was inhibited. However, in some cases no inhibition of microbial growth ware observed. The absence of activity could be due to the evaporation of the active compounds or alternatively due to very little amount of the active compound (Shahverdi *et al*., 2007).

Most of the antifungal agents detected in this study were present in extracts of relatively non-polar solvents such as chloroform. These findings are in good agreement with the previously published results (Masoko and Eloff, 2005) that showed the substances responsible for the antimicrobial activity were mainly non-polar in nature. However, the ethanol and butane crude extracts of *Trichoderma* isolates, in contrast to the chloroform crude extracts, had good activity against plant pathogenic fungus (Shahverdi *et al*., 2007). AUT5 had the highest number of inhibition spots (12) against the test fungus, while AUT6 had the lowest number of inhibition spots (8) against the test fungus. It is important to note that bioautography is not a quantitative measure of antifungal activity, since it only indicates the number of compounds that were separated with antifungal activity.

With regard to the antagonistic activity of culture filtrates, the inhibition varied depending on the *Trichoderma* isolates producing the metabolites from 18.9% to 78.4% (Fig. 2) in non-volatile compounds on PDA culture media after 7 days of incubation at 25°C. *Fusarium xylarioides* growth was significantly reduced in the presence of metabolites produced by isolates AUT2, AUT3

and AUT4 than the other isolates of *Trichoderma*. It has also been observed that the crude extracts and culture filtrates from all isolates of *Trichoderma* have antagonistic effect towards the test pathogen. Similar observations have been reported by various authors including Hassan *et al*. (2011), who demonstrated that the crude extract of antifungal compounds was active against *F. oxysporum*.

CONCLUSIONS

In the *in vitro* bioassay, the highest mean inhibitory effect against the growth of the pathogen was achieved by AUT2 isolate in dual culture. The optimum R_f value was obtained by only three extraction solvents; *viz*., chloroform, ethanol and butane out of the seven pre-screened solvent systems. Bioautographic method was found to be very useful in isolating active compounds with antifungal activity since the R_f value of the active compounds can be used in bioassay guided fractionation instead of requiring labour intensive determination of activity of the crude extracts. TLC-direct bioautography tests revealed that most of the crude extracts from *Trichoderma* isolates exhibited antifungal activity toward *F. xylarioides*. The best extraction solvent for the active antifungal compounds was found to be chloroform.

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